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ORIGINAL ARTICLE

Dual Isoflurane-induced Preconditioning Improves Neuroprotection in Rat Brain *In Vitro* and the Role of Extracellular Signal-regulated Protein Kinase[△]

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Objective To test the ability of isoflurane-induced preconditioning against oxygen and glucose deprivation (OGD) injury *in vitro*.

Methods Rat hippocampal slices were exposed to 1 volume percentage (vol%), 2vol% or 3vol% isoflurane respectively for 20 minutes under normoxic conditions (95% O₂/5% CO₂) once or twice (12 slices in each group) before OGD, with 15-minute washout after each exposure. During OGD experiments, hippocampus slices were bathed with artificial cerebrospinal fluid (ACSF) lacking glucose and perfused with 95% N₂ and 5% CO₂ for 14 minutes, followed by a 30-minute reperfusion in normal ACSF. The CA1 population spike (PS) was measured and used to quantify the degree of neuronal function recovery after OGD. To assess the role of mitogen-activated protein kinases (MAPKs) in isoflurane preconditioning, U0126, an inhibitor of extracellular signal-regulated protein kinase (ERK1/2), and SB203580, an inhibitor of p38 MAPK, were used before two periods of 3vol% isoflurane exposure.

Results The degree of neuronal function recovery of hippocampal slices exposed to 1vol%, 2vol%, or 3vol% isoflurane once was 41.88%±9.23%, 55.05%±11.02%, or 63.18%±10.82% respectively. Moreover, neuronal function recovery of hippocampal slices exposed to 1vol%, 2vol%, or 3vol% isoflurane twice was 53.75%±12.04%, 63.50%±11.06%, or 76.25%±12.25%, respectively. Isoflurane preconditioning increased the neuronal function recovery in a dose-dependent manner. U0126 blocked the preconditioning induced by dual exposure to 3vol% isoflurane (6.13%±1.56%, *P*<0.01) and ERK1/2 activities.

Conclusions Isoflurane is capable of inducing preconditioning in hippocampal slices *in vitro* in a dose-dependent manner, and dual exposure to isoflurane with a lower concentration is more effective in triggering preconditioning than a single exposure. Isoflurane-induced neuroprotection might be involved with ERK1/2 activities.

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THERE is an increasing interest in the phenomenon of anesthetic preconditioning (APC) in both basic science and clinical research in brain. Evidence suggested volatile anesthetics may exert a neuroprotective effect when given before and after ischemia.¹⁻³ The neuroprotective effect of APC is identical to ischemic preconditioning (IPC).⁴

APC has the advantage of not requiring ischemia to initiate preconditioning. It has been shown that repeated transient ischemia-reperfusion (IR) periods were more effective than a single IR period in triggering IPC.^{5, 6} Although neuroprotection induced by single and repeated exposure to APC has been found to be dose-dependent,^{1, 7} there has been no reported comparison of repeated with single anesthetic exposure in improving neuroprotection. This study investigated whether dual exposure to isoflurane was more effective in triggering APC in hippocampal slices than a single exposure and whether the use of a lower concentration with lesser side effects would achieve an outcome similar to that associated with a single exposure to a higher concentration.

The neuroprotective effect of APC and IPC seems to act largely through the same signal transduction pathways. This effect is believed to be achieved through complex signal transduction pathways involving activation of adenosine receptors,^{8, 9} protein kinase C,^{10, 11} and mitochondrial adenosine triphosphate-regulated potassium (K_{ATP}) channels.^{1, 7} Mitogen-activated protein kinases (MAPKs), one of the most ancient signaling molecules, involved in a vast array of physiological processes.¹² Extracellular signal-regulated protein kinase (ERK1/2) and p38 MAPK activities are important in the genesis of the cytoprotective phenotype either by ischemia or by pharmacologic agents.^{13, 14} However, no data are available at present with respect to the role of MAPKs in APC. Therefore, apart from comparing the effect of dual APC with that of single one, this study also aimed to determine the significance of ERK1/2 and p38 MAPK signaling pathways in APC-induced neuroprotection and to characterize the activity profile of these kinases in APC.

MATERIALS AND METHODS

Materials

Isoflurane, a volatile anesthetic obtained from Dinabot Co. Ltd. (Osaka, Japan), is routinely used for general anesthesia, and compared with other preconditioning drugs through intravenous administration the concentration and duration of action in the body are easy to control. Most of the chemicals were obtained from Sigma (St. Louis, Mo., USA), except for ERK1/2 inhibitor U0126 and p38 MAPK inhibitor SB203580 (Promega Co., Madison, Wis., USA). U0126 (10 μ mol/L) and SB203580 (2 μ mol/L) were dissolved in dimethyl sulfoxide (DMSO) and diluted in cold artificial cerebrospinal fluid (ACSF) before they were perfused into the chamber. ACSF composed of 124 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1.25 mmol/L NaH_2PO_4 , 2 mmol/L $MgSO_4$, 26 mmol/L $NaHCO_3$, and 10 mmol/L glucose, dissolved in high purity (18 M Ω /cm²) deionized water. The ACSF was pre-cooled to 0°C-4°C and saturated with a 95% O₂/5% CO₂ gas mixture before use (pH 7.3-7.4).

Hippocampal slice preparation

Upon approval from the Animal Research Committee of Shihezi Medical College, male Sprague Dawley rats (weight, 100-120 g) supplied by Shihezi Medical College were decapitated during ethyl ether anesthesia. Hippocampal slices, one from each rat, were prepared following the methods previously described by Hirota and Roth.¹⁵ Briefly, the brain was rapidly removed and immersed in cold ACSF saturated with 95% O₂/5% CO₂. The dissected hippocampus was sliced into 400 μ m sections transversely to their longitudinal axis by a Rotorslicer DTY-7700 (DSK, Osaka, Japan). The slices were placed on a nylon mesh in a recording chamber at 34°C. A humidified gas mixture (95% O₂/5% CO₂) was injected to the chamber at a rate of 1 L/min. ACSF was continuously infused at a rate of 1.5 mL/min. Slices were stabilized in this environment for 90 minutes without electrical stimulation.

Drug

The general anesthetic, isoflurane, was applied as vapors to ACSF *via* the pre-warmed and humidified 95% O₂/5%

CO₂ gas stream, using the appropriate vaporizer Tec 3 (Ohmeda, Steeton, West Yorkshire, UK). Concentration of isoflurane expressed as volume percentage (vol%) refers to dial settings on the vaporizer, which had been previously calibrated with an anesthetic gas analyzer (Capnomac, Datex, Helsinki, Finland). The ACSF was saturated with isoflurane for at least 20 minutes before it was perfused into the chamber, as the preliminary experiments demonstrated that the onset time for isoflurane effects was less than 2 minutes and that the maximum effects were reached a plateau within 10 minutes (data not shown). Concentrations of isoflurane in the perfusate of the recording chamber were determined using gas chromatography (Shimazu, Kyoto, Japan). The concentrations of isoflurane in ACSF determined using gas chromatography showed a linear relationship with the volume percentage (0.55 mmol/L per 1vol%) within 5.0vol%.

Experimental protocol

To investigate the effect of isoflurane administration before oxygen and glucose deprivation (OGD) on neuroprotection, the stabilized slices were randomly assigned to seven groups (12 slices in each group): control, Iso1-1, Iso1-2, Iso2-1, Iso2-2, Iso3-1, and Iso3-2 groups. The slices in Iso1-1, Iso2-1, or Iso3-1 groups were exposed to 1vol%, 2vol%, or 3vol% isoflurane for 20 minutes respectively, before 14-minute OGD period. The slices in Iso1-2, Iso2-2, or Iso3-2 groups were exposed to two periods of 1vol%, 2vol%, or 3vol% isoflurane respectively, with 15-minute washout after each exposure.

To assay the role of MAPKs in isoflurane preconditioning, the stabilized slices were randomly assigned to the following seven groups (12 slices in each group): control, Iso, U0126, SB203580, Iso+U0126, Iso+SB203580, and vehicle groups. Iso group was exposed to two episodes of 3vol% isoflurane for 20 minutes interspersed with 15-minute washout period. U0126 and SB203580 groups were subjected to 10 μ mol/L U0126 and 2 μ mol/L SB203580 respectively dissolved in ACSF for 10 minutes before OGD. Iso+U0126 and Iso+SB203580 groups were exposed to 10 μ mol/L U0126 and 2 μ mol/L SB203580 respectively dissolved in ACSF for 10 minutes followed by two periods of 3vol% isoflurane exposure. Control was not exposed to either isoflurane, U0126 or SB203580. The slices in vehicle group were incubated with 1 mmol/L DMSO dissolved in ACSF.

During the OGD, the slices were maintained using glucose-free ACSF and gassed with the mixture of 95% N₂/5%CO₂ for 14 minutes. The OGD period was followed by a 30-minute reoxygenation period. Control group received no pretreatment before 14-minute OGD.

Electrophysiological analysis

Bipolar nichrome stimulating electrodes (0.1 ms pulse width, 8-9 V in intensity, Nihon Kohden, Tokyo, Japan) were placed in the region of CA3 to activate Schaffer collateral (orthodromic stimulation) inputs to CA1 pyramidal neurons. Glass microelectrodes (3-5 M Ω , filled with 2 mmol/L NaCl) were placed in the cell body region of CA1 neurons to extracellularly record the CA1 population spike (PS). The stimulation intensity (8-9 V) that elicited the maximum PS amplitude was used. Responses were automatically recorded every 10 seconds after washout of anesthetic-equilibrated ACSF from the chamber. Square-wave stimuli (8-9 V, 0.1 ms, 0.1 Hz) were delivered using a SEN-3301 stimulator (Nihon Kohden, Tokyo, Japan). Field potentials were obtained using Nihon Kohden MEZ-8301 amplifier. Data were filtered (1 Hz to 10 kHz), converted at 100 kHz using an iNet system (GWI, Somerville, MA, USA), and stored on a computer for later analysis. Only the slices in which orthodromic stimulation evoked a PS response of more than 3 mV were qualified for further investigations.

The PS amplitude was defined as the amplitude from the first positive peak to the succeeding negative peak. The degree of recovery of neuronal function after OGD was expressed as the ratio of PS at the end of reoxygenation to PS after washout of isoflurane.

In this study, the concentrations of isoflurane that depressed PS amplitudes more than 50% of control in normal (ACSF) condition were used. The stimulus intensity was adjusted in each experiment to produce PS amplitude of 50% of the maximum responses.

Propidium iodide (PI) staining

To assess the viability of neuron, after the OGD period the slices were reperfused in 30 mL ACSF gassed with 95% O₂/5% CO₂ for 30 minutes. At the end of the reperfusion, the slices were stained with PI (2 μ g/mL) for 30 minutes at 37°C. PI fluorescence intensity was observed *via* a reverse fluorescence microscope equipped with filter (excitation wavelength at 530 nm, emission wavelength>600 nm; Leica, San Francisco, CA, USA) and images were photoed with digital camera. All the images were analyzed with Photoshop 7.0 image processing software, then death cells were quantified by measuring mean fluorescence intensity (MFI) of PI staining.¹⁶

Statistical analysis

The data were all drawn from at least three replications. Inter-group comparisons of neuronal function were performed with one-way analysis of variance (ANOVA) followed by post hoc Duncan test (SPSS 11.0). Intra- and

inter-group differences were considered statistically significant when the P value was less than 0.05. All data were expressed as mean $\pm$ SEM.

RESULTS

In control group, PS amplitude at the start of the experiment was 6.20 ± 0.80 mV. After OGD period, the PS amplitude was declined to the baseline level and showed a slight recovery to 0.28 ± 0.06 mV during the reoxygenation period ($4.75\%\pm1.39\%$ of the control PS amplitude at the start of experiment). In the other groups, the PS amplitudes at the start of experiment were not significantly different from the control group ($P>0.05$). DMSO used did not affect the PS amplitudes in the experiments. In vehicle group, the degree of neuronal function recovery was $5.28\%\pm1.53\%$.

Effects of isoflurane preconditioning on neuronal function recovery

The neuronal function recovered of control group following 14-minute OGD period and 30-minute reoxygenation was $4.75\%\pm1.39\%$. The degree of neuronal function recovery of hippocampal slices exposed to 1vol%, 2vol%, or 3vol% isoflurane once was $41.88\%\pm9.23\%$, $55.05\%\pm11.02\%$, or $63.18\%\pm10.82\%$, respectively. Moreover, neuronal function recovery of hippocampal slices exposed to 1vol%, 2vol%, or 3vol% isoflurane twice was $53.75\%\pm12.04\%$, $63.50\%\pm11.06\%$, or $76.25\%\pm12.25\%$, respectively. Isoflurane preconditioning increased the neuronal function recovery in a dose-dependent manner. The neuronal function recovery of isoflurane pretreatment groups was significantly higher than that of control (all $P<0.01$).

Dual exposure to isoflurane at the same concentration was more effective in preconditioning than single exposure. There was a significant difference in neuronal function recovery between Iso1-2 and Iso1-1 groups ($P<0.05$), as well as between Iso3-2 and Iso3-1 groups ($P<0.05$). At the same time, we found that duplicate exposure to a lower concentration of isoflurane achieved an outcome similar to a single exposure to a higher concentration. There was no significant difference in neuronal function recovery between Iso1-2 and Iso2-1 groups ($P>0.05$), also between Iso2-2 and Iso3-1 groups ($P>0.05$).

Role of ERK1/2 and p38 MAPK signaling pathways in APC-induced neuroprotection

In all isoflurane groups, the variability of PS amplitudes during the initial data acquisition period and following washout of anesthetics was less than 5%. Changes in ex-

tracellular PS during and following ischemia (14-minute OGD and 30-minute reoxygenation) in hippocampal slices from control, Iso3-2, Iso+U, and Iso+SB groups are shown in Figure 1.

ERK1/2 inhibitor U0126 or p38 MAPK inhibitor SB203580 alone had no effect on the neuronal function recovery ($6.12\%\pm1.58\%$ and $5.88\%\pm1.80\%$). The neuronal function recovery of hippocampal slices exposed to two episodes of 3vol% isoflurane was $76.25\%\pm12.25\%$. However, U0126 blocked the preconditioning induced by dual exposure to 3vol% isoflurane ($6.13\%\pm1.56\%$, $P<0.01$). SB203580 had no effect on the preconditioning induced by dual exposure to 3vol% isoflurane ($55.88\%\pm6.58\%$, $P>0.05$).

Effects of isoflurane preconditioning on neuronal viability

PI staining showed OGD significantly increased death cells in the hippocampal slices (MFI: 63.6 ± 13.1 , $P<0.01$, Fig. 2).

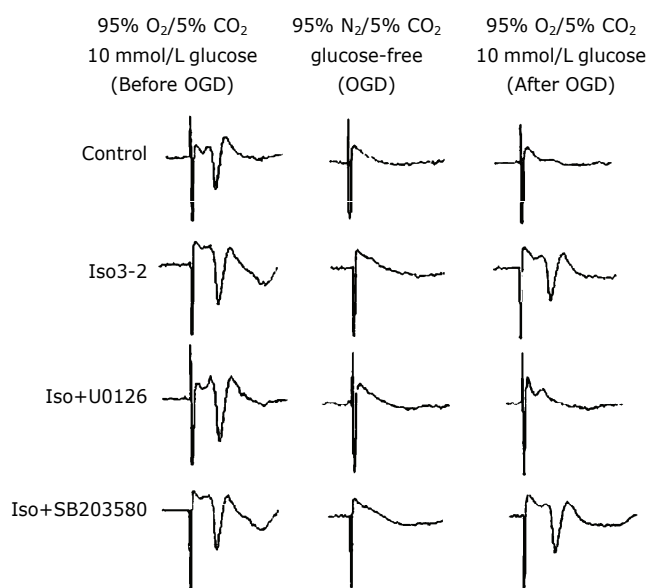


Figure 1. Extracellular population spike (PS) traces in control, Iso, Iso+U0126, and Iso+SB203580 groups before, during, and after oxygen and glucose deprivation (OGD).

In control group, the PS recovers only a little after OGD; in Iso group (preconditioning with 3vol% isoflurane twice), the PS amplitude is larger than that in control group after OGD; in Iso+U0126 group, the PS only recovers slightly after OGD, showing U0126 ($10\text{ }\mu\text{mol/L}$) abolish the effect of preconditioning induced by dual exposure to 3vol% isoflurane; and in Iso+SB203580 group, the changes of amplitude are similar with those in Iso3-2 group, showing SB203580 ($2\text{ }\mu\text{mol/L}$) has no influence on the effect of preconditioning induced by dual exposure to 3vol% isoflurane.

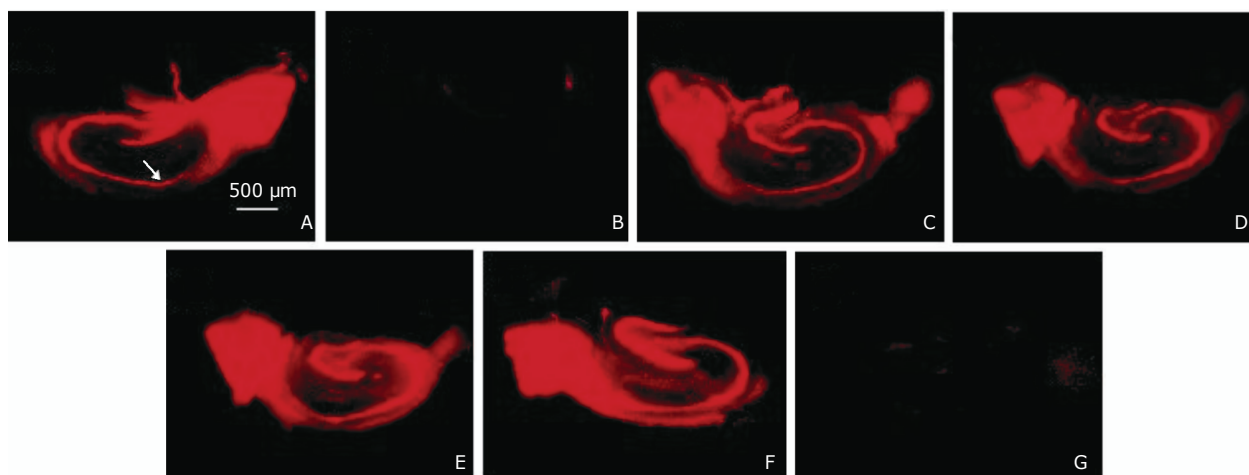


Figure 2. Microscopic fluorescence images of hippocampal slices stained with propidium iodide.

Images were acquired 30 minutes after the simulated ischemia. Bright areas indicate propidium iodide fluorescence (dead neurons). Arrow indicates CA1 area.

A. control group; B. Iso group; C. U0126 group; D. SB203580 group; E. vehicle group; F. Iso+U0126 group; G. Iso+SB203580 group.

Dual exposure to 3vol% isoflurane ($\text{MFI: } 7.1 \pm 1.0$, $P < 0.01$) could significantly decrease the number of damaged cells compared with OGD insult alone. We observed similar changes in MFI of hippocampal slices treated with U0126 (68.7 ± 15.3) and SB203580 (70.5 ± 16.1) alone compared to those with OGD injury ($P > 0.05$). The neuronal protection effect of isoflurane could be attenuated by U0126 ($\text{MFI: } 69.8 \pm 11.7$, $P < 0.01$, Fig. 2), but not be reduced by SB203580 ($\text{MFI: } 11.7 \pm 1.4$, $P > 0.05$, Fig. 2).

DISCUSSION

The current study showed several salient findings. First, isoflurane-induced preconditioning *in vitro* was dependent not only on the concentration but also on the preconditioning cycles. Second, the neuroprotective effects of APC could be improved by two cycles of exposure compared with a single one in the same isoflurane concentration. Finally, administration of U0126, a specific MEK-ERK1/2 inhibitor, reversed the neuroprotective effects induced by 3vol% isoflurane preconditioning. Collectively, the data presented provide direct evidences that isoflurane preconditioning could induce short-term neuroprotection against OGD injury *via* activating MEK-ERK1/2 inhibitor.

The mechanisms of anesthetics preconditioning have not been fully illustrated. Hippocampus may be one of the major targets for anesthetic action.¹⁷ Recently, studies suggested that preconditioning with isoflurane could induce neuroprotection in rats hippocampal slice cultures¹⁸ and neurons.¹⁹ These results suggested isoflurane preconditioning could induce long-term neuroprotection. In our present study, we chose PS and MFI of PI staining to

investigate the neuroprotective effects of isoflurane preconditioning on CA1 area of hippocampal slices. The neuronal function could be well indicated by PS amplitude, and the change of amplitude stands for the change of the neuronal function.²⁰ Meanwhile, MFI of PI staining could stand for the vitality of neurons.¹⁶ Hippocampus may not be the sole area achieving the neuroprotection induced by isoflurane preconditioning. It has been reported that isoflurane preconditioning induced neuroprotection in a model of global cerebral ischemia in rats.¹⁶ Hippocampus may not be the sole area achieving the neuroprotection induced by isoflurane preconditioning. It has been reported that isoflurane preconditioning induced neuroprotection in a model of global cerebral ischemia in rats.^{21,22} In another report, Xiong *et al*¹ found that pretreatment with 1.50% and 2.25% isoflurane respectively for 1 hour per day for 5 days in oxygen could induce ischemic tolerance in rat focal cerebral ischemia model in a dose-dependent manner. All these results demonstrated that isoflurane preconditioning might play an important therapeutical role against cerebral ischemia. Some of our results were consistent with these studies, and the difference was that these studies did not investigate the effects of repetitive exposures to anesthetic *in vitro* and the relationships between single and repeated exposure to anesthetic. The present investigation confirmed these results by using different protocols (one or two cycles of exposure to isoflurane). It has been shown that repeated transient IR periods were more effective in triggering IPC than a single IR in brain.⁵ This suggested greater accumulation of critical IPC mediators with each IR period. The mechanisms of APC may be similar to IPC. Our results suggested at least two cycles of exposure to a clinically relevant concentration of volatile anesthetic might enhance neuroprotection afforded by APC. This outcome may be achieved *via* the accumulation of the mediators. But whether those two types of preconditioning are sharing

the same mechanisms need to be further elucidated.

The present study revealed that U0126, a specific MEK-ERK1/2 inhibitor, abolished the neuroprotective effects induced by two cycles of exposure to 3vol% isoflurane preconditioning. Previous evidence indicated that U0126 could block the neuroprotection induced by isoflurane in hippocampal slice cultures.²³ In that study, isoflurane and the hypoxic gas entered the incubator chamber through a calibrated vaporizer at the same time, their results suggested the neuroprotection of isoflurane was induced by activating the MEK-ERK1/2 signal pathway, but the neuroprotective effect was not induced by anesthetic preconditioning. We presumed that effects of preconditioning induced by isoflurane might be through activating this signal pathway, and our results confirmed the hypothesis. MEK-ERK1/2 links growth factor receptors with nerve growth factor and brain-derived neurotrophic factor, to exert neuroprotective effects such as NMDA receptor modulation and reduction of glutamate excitotoxicity.²⁴ MEK-ERK1/2 also phosphorylates hypoxia-inducible factor 1 α , a transcription factor required for the adaptation of cells to acute and chronic hypoxia.^{13,17} We found that p38 MAPK had no effect on the preconditioning of isoflurane. The role of p38 MAPK in preconditioning is disputed, Zheng *et al*²⁵ found that isoflurane induced a rapid and prolonged increase of the phosphorylated p38 MAPK in cerebral neocortex. These results suggested that isoflurane preconditioning may improve long-term neurological outcome after focal brain ischemia and that the effects may be mediated by activating p38 MAPK. Jiang *et al*¹⁴ reported that brain seizure tolerance involved p38 signaling pathways. In contrast, Sugino *et al*⁶ demonstrated that pretreatment with SB203580 could reduce ischemic cell death in the CA1 region after transient global ischemia by inhibiting the activity of p38 MAPK. Unfortunately, there is no easy explanation for the opposing conclusions of the two studies which used almost identical models.

The protective actions of isoflurane on signaling pathways are not in conflict with earlier proposals that isoflurane either inhibits excitotoxic^{26, 27} or augments inhibitory processes.²⁸ The signaling mechanisms described here interact with processes involved in excitotoxicity. For example, activation of the MAPK ERK pathway *via* growth factor receptors results in decreased NMDA receptor activity and excitotoxicity.²⁴ It is possible that one neuroprotective effect of isoflurane is related to second-messenger modulation of NMDA receptors by cross-talk.

There has been shortage in our investigations: the isolated hippocampal slice is devoid of hormonal input, which may influence IR injury. Systemic storage of the

anesthetic *in vivo* likely prolongs wash-in and washout time compared with our model. Clearly, more studies are needed to identify the mechanisms of protection afforded by repeated exposures compared with a single exposure to a volatile anesthetic and to ultimately find an optimal protocol to induce APC.

In summary, the present results demonstrated that both one and dual exposure to isoflurane induced neuroprotection against OGD injury in the rat hippocampus slices preparation *in vitro* by APC were dose-dependent. Two cycles of exposure to isoflurane improved the effect of preconditioning induced by APC. Activation of MEK-ERK1/2 might be a key mechanism of isoflurane-induced preconditioning. These findings suggest that administration of volatile anesthetics preoperatively might achieve cerebroprotection in humans.

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